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Effects of Hyperbaric Pressure on Tissue Phospholipid Metabolism

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Abstract

We have been pursuing the characterization of subcellular and tissue culture preparations prior to initiating hyperbaric pressure studies. We have been successful in applying free-flow electrophoresis to the further purification of synaptosomes and lysosomes, after initial purification by centrifugation. These are being studied for phospholipid composition and enzymatic activity, including the phospholipases. We have succeeded in culturing and establishing a culture line of astrocytes in our tissue culture facility. Immunocytochemical studies are under way for morphological characterization of these cells. The endothelial cell culture system has also been established and additional morphological endpoints, including antibody techniques, are being developed prior to extending this work to the hyperbaric pressure studies. (AW)

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Research Goals:

At the initial planning meeting on May 26, 1988 with the NMRI staff, we set the following priorities for the pursuit of the research on *Effects of Hyperbaric Pressure on Tissue Phospholipid Metabolism*: "The initial discussions of the research plan centered around the studies of neurosynaptic vesicles and lysosomes and the application of free-flow electrophoresis to the purification of these subcellular membrane systems....for studies in which highly purified lysosomes and neurosynaptic vesicles are required, this may be a major final purification step prior to stressing these vesicles with hyperbaric pressure... The second phase of the meeting was devoted to cellular models, including cultured endothelial cells and cultured astrocytes... Drs. Dickens, Goldberg, and Bernstein presented information about the present tissue culture core facility at George Washington University Medical Center in which endothelial and smooth muscle cells are being grown: plans for transfer of an astrocyte culture from the VA hospital to this facility are under way... Priorities were discussed, with immediate plans for collaborative work to involve the neurosynaptic vesicle purification and analyses of phospholipids and other markers of potential contamination of this preparation."

Progress Report for 1988:

We were able to begin the above work in August, 1988 because of the delay in activation of funding. During this past summer we have been successful in recruiting Dr. Richard Stafford, who has been able to concentrate on lysosomal, synaptosomal and neurosynaptic vesicle purification and characterization. Assisting him in this work is Ms. Mary Reinhold, a new research technician. Dr. Sarah Gilman and her technician at NMRI have been preparing brain synaptosomes from the guinea pig model and transporting them to George Washington University Medical Center. The free-flow electrophoresis unit has been utilized on an almost daily basis, devoted either for purification of the synaptosomes or neurosynaptic vesicle preparation or the tissue lysosomes. The feasibility of using this novel electrophoretic preparation has been established and this report will outline the progress that has been made over the past five months in this regard (see Addendum 1).

Since our initial planning meeting in May of 1988. Drs. Dickens, Bernstein and Goldberg have established the astrocyte culture model in the tissue culture core facility in the Division of Experimental Medicine at George Washington University Medical Center. These cells are now well established and have been characterized morphologically (see Addendum 2). Selected antibodies have been obtained for immunocytochemical studies. In addition to the astrocytes, the endothelial cells have been utilized on a regular basis as well. We have developed this model for studying injury induced by exogenous administration of a free radical generation system using dihydroxyfumarate-iron ADP. The techniques for phospholipid analyses and measurements of loss of cellular integrity have been standardized. We have been able to develop the use of spin trapping agents that characterize the free radicals in the aqueous and membrane phases of our membrane preparations. This methodology has been used to assess the effects of anti-radical pharmacological agents on the intensity of radical generation in the aqueous phase as well as the ability of these agents to block peroxidative changes within membrane systems. Thus, the research has reached the point of applicability to the lysosome/neurosynaptic vesicle organelles as well as the endothelial/astrocyte models.

Planned Research for 1989:

We will continue to obtain more than 40-fold pure hepatic lysosomes using the free flow electophoresis unit for in-vitro studies. The phospholipases of these lysosomes will be characterized by thin layer and HPLC chromatographic techniques. Radiolabeled phospholipid substrates for the characterization of the phospholipases of these lysosomes have been ordered. Our new Beckman liquid scintillation counter is "up and running" for conducting these assays in the months ahead. In our search of the literature we have not found substantial data characterizing the phospholipases of the neurosynaptic vesicle preparation, so that we are looking forward to obtaining this data in the coming year. The structural phospholipid analyses are under way and the characterization of the classes of phospholipids is progressing nicely. Both subcellular organelle preparations will be transported to the Bethesda NMRI facility for hyperbaric experiments; however, the existing chamber is undergoing repairs at present. The endothelial and astrocyte cell preparations will also be utilized for characterization of phospholipid and phospholipase activity, prior to exposure to hyperbaric pressure and decompression. We have recruited a half-time research associate to assist in the morphological studies. The morphological characterization of the normal cells will be completed prior to utilizing this methodology to assess the effect of hyperbaric pressure on structural parameters. We anticipate that these hyperbaric intervention experiments will begin in the spring of 1989.

Appendix 1. Isolation and characterization of Lysosomes and Synaptosomes:

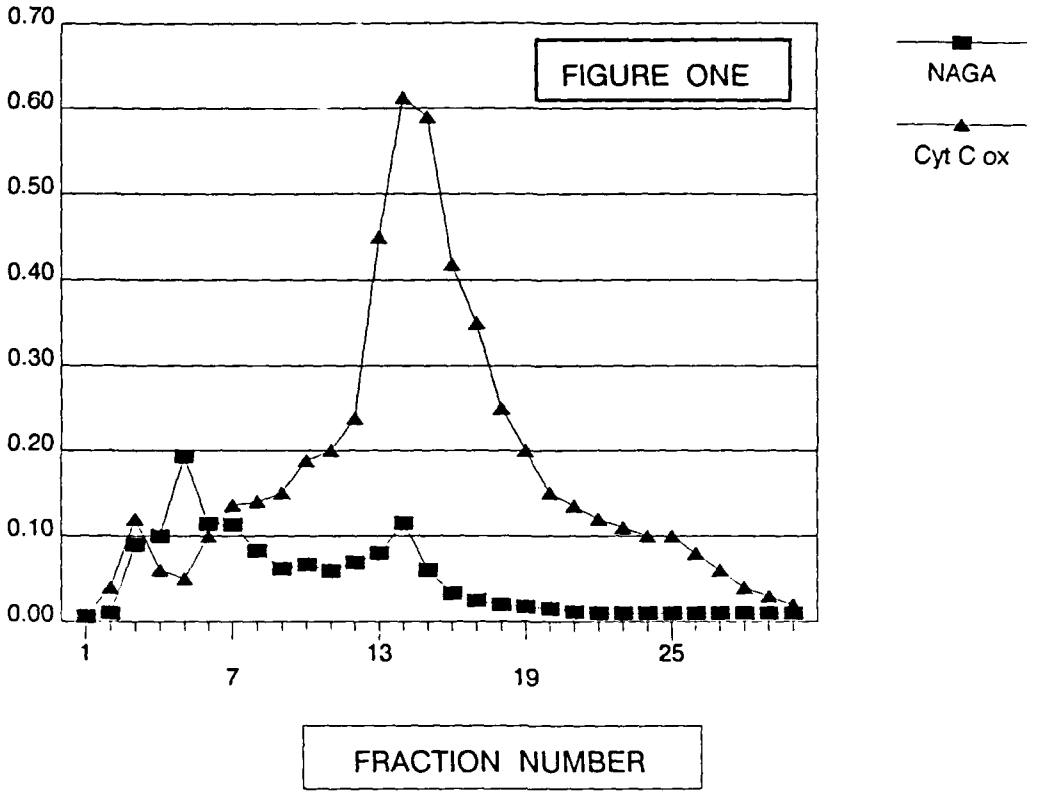
Lysosomes from rat liver have been successfully isolated and purified by homogenization, differential centrifugation and subsequent purification by free flow electrophoresis. Figure 1 shows a profile of free flow electrophoresis fractions of lysosomes contaminated with mitochondria. The lysosomes are identified by the enzyme N-acetyl glucosaminadase and the mitochondria are identified by cytochrome C oxidase. The lysosome fractions obtained by free flow electrophoresis were enriched 40 times over the crude liver homogenate.

Our next step is to profile phospholipase activity of the purified lysosomes. These profiles will be done at standard pressure and at hyperbaric pressures. We have just recieved approval to use radioactive materials therefore the phospholipases will be assayed with both exogenous radioactive substrates and by observing changes in endogenous substrates.

Synaptosomes isolated from guinea pig brain homogenates by sucrose density gradient centrifugation were further purified by free flow electrophoresis. A profile of the free flow electrophoresis fractions are shown in Figure 2. Figure 2 shows that the synaptosomes which are identified by the enzymes cytochrome C oxidase and acetyl choline esterase are separated from other proteinatious materials and phospholipids. The peak synaptosome fraction which corresponds to the second peak of acetyl choline esterase activity (fraction 12) was enriched 10 times over the synaptosome isolated by sucrose gradient centrifugation. The first peak of acetyl choline esterase activity probably corresponds to membrane fragments, but no morphology has been done on these fractions to confirm this.

The synaptosome lipids will now be identified and quantitated using TLC with differential lipid stains, HPLC, and mass spectrometry. After the lipids are analyzed the lipase activities will be profiled using both exogenous and endogenous substrates at standard and hyperbaric pressures.

The lipids of C6 cells which are derived from astrocytes are being analyzed by TLC using differential lipid stains. Once the lipid have been identified and quantitated the cells lipase activities will be profiled.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0.60
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13
19
25
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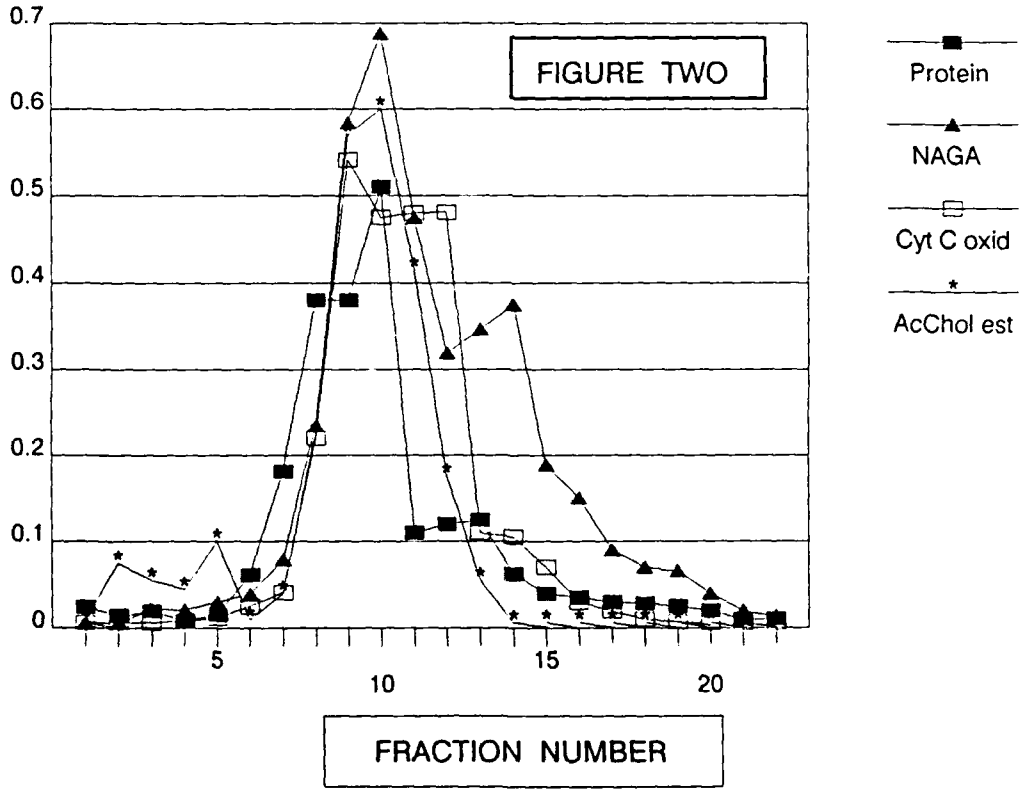


FIGURE TWO
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
5
10
15
20
FRACTION NUMBER
Protein
NAGA
Cyt C oxid
AcChol est

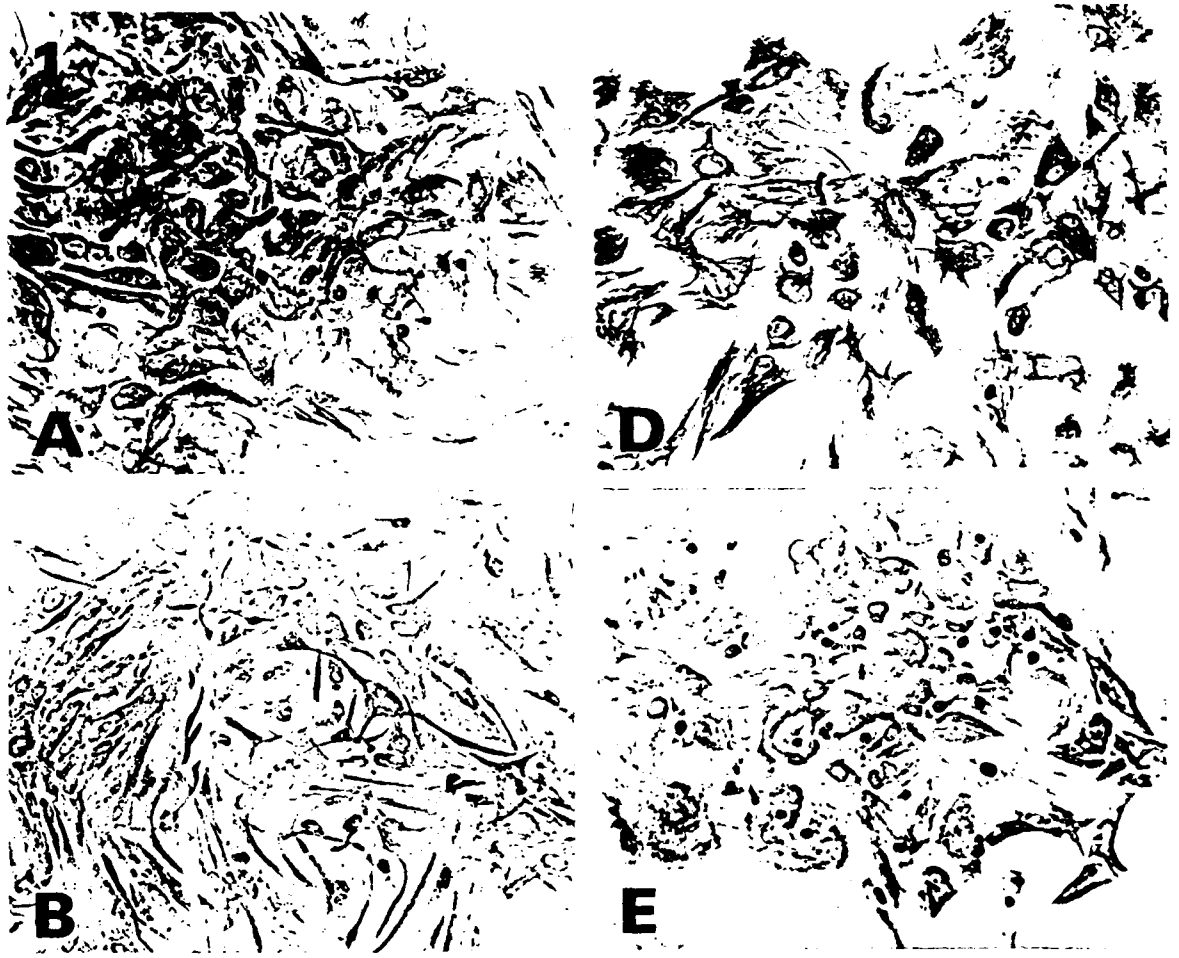
Appendix 2 Morphological Characterization of Cultured Astrocytes

Figure 1.

Immunohistochemistry of cultured fetal astrocytes from rat cerebral cortex (A-C) and basal ganglia (D-H). Cultures were stained for the presence of glial fibrillary acidic protein (A and D) or vimentin (B and E). Negative controls (omission of primary antibody) are in C and F. Low and high power scanning electron micrographs of basal ganglia astrocytes are in G and H, respectively. The cell in H (white arrow) is also marked by a white arrow in G.

Figure 2.

Light and electron microscopic analysis of C6 cells (transformed rat astrocyte cell line) in culture. A. Cultures stained for the presence of glial fibrillary acidic protein (GFAP) indicate that C6 cells synthesize GFAP, a specific marker for cells of astrocytic origin. B. C6 cells also label positively for the presence of vimentin. The vimentin reaction is more uniform than that observed for GFAP. C. Negative control (omission of primary antibody) observed under phase contrast optics. D. Low power scanning electron micrograph of cultured C6 cells. E. Transmission electron micrograph of cultured C6 cells. F. High power scanning electron micrograph of cultured C6 cells.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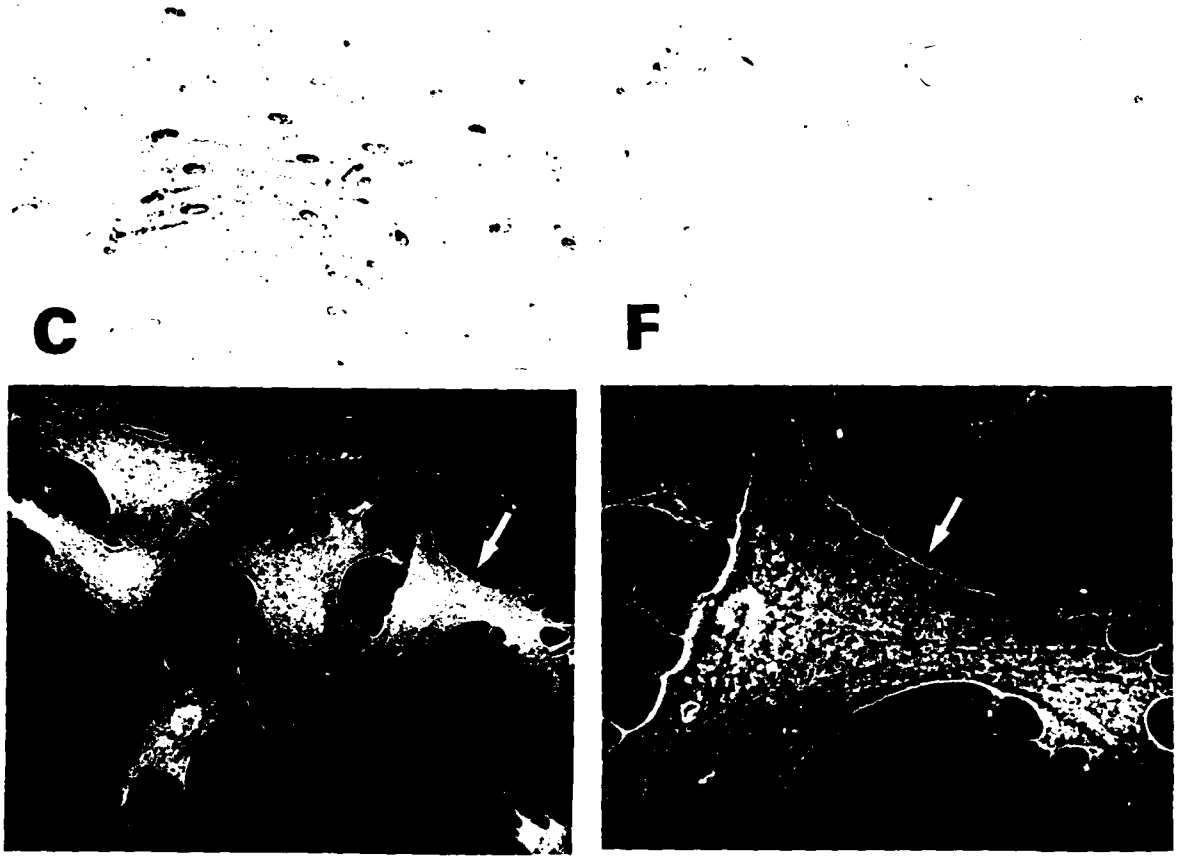
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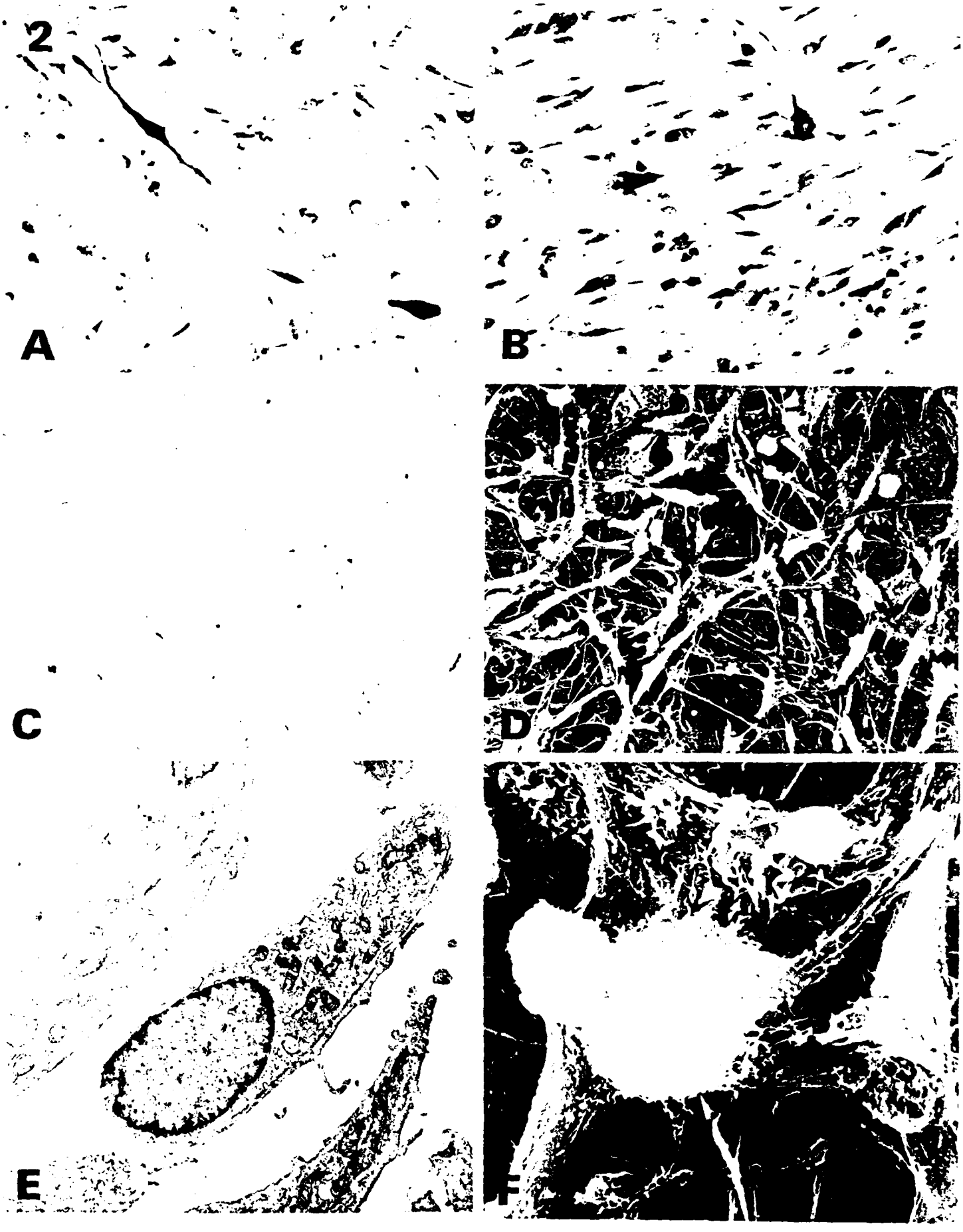
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